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Intercellular Communication in In Vivo- and In Vitro-Produced Bovine Embryos

R. Boni,[1,2] E. Tosti,[3] S. Roviello,[3] and B. Dale[3]

Dipartimento di Scienze delle Produzioni Animali,[2] 85100 Potenza, Italy
Stazione Zoologica 'Anton Dohrn',[3] Villa Comunale, 80121 Napoli, Italy

ABSTRACT

In vivo bovine embryos were obtained by nonsurgical flushing of uterine horns of cows submitted to superovulatory treatment, while in vitro embryos were generated from oocytes collected from slaughtered donors. Lucifer Yellow injected into single blastomeres did not diffuse into neighboring cells until the morula stage in in vivo embryos and the blastocyst stage in in vitro embryos. In both cases diffusion was limited to a few cells. In contrast, diffusion was extensive in microsurgically isolated inner cell mass (ICM) but absent in the trophectoderm (TE). At the blastocyst stage, diffusion was always more extensive in in vivo than in in vitro embryos. Ultrastructural analyses confirmed these functional observations, and gap junction-like structures were observed at the blastocyst stage. These structures were diffuse in the ICM of in vivo embryos, scarce in the ICM of in vitro embryos and in the TE of in vivo embryos, and not observed in the TE of in vitro embryos. Blastomeres at all stages of development from the 2-cell stage to the blastocyst stage in in vitro embryos and at the morula and blastocyst stage in in vivo embryos were electrically coupled, and the junctional conductance (Gj) decreased in in vitro embryos from 4.18 ± 1.70 nS (2-cell stage) to 0.37 ± 0.12 nS (blastocyst stage). At each developmental stage, in vivo embryos showed a significantly ($P < 0.05$) higher Gj than in vitro-produced embryos. Moreover, a significantly ($P < 0.01$) higher Gj was found in isolated ICM than in the respective blastocyst in both in vivo- and in vitro-produced embryos (3.5 ± 1.4 vs. 0.7 ± 0.3 and 2.6 ± 1.6 vs. 0.37 ± 0.12 nS, respectively). The electrical coupling in absence of dye coupling in the early bovine embryo agrees with observations for embryos from other phyla. The late and reduced expression of intercellular communicative devices in in vitro-produced embryos may be one of the factors explaining their developmental low efficiency.

INTRODUCTION

Over the last decade, in vitro embryo production (IVEP) in cattle has improved remarkably [1]. Further improvements are needed in order to commercially apply this technology globally. The efficiency of IVEP in terms of in vitro culture of in vitro-matured and -fertilized embryos is satisfactory, since the same number of embryos are obtained whether they have been cultured in vitro, or in vivo in the ligated oviducts of foster animals [2, 3]. A significant improvement in embryo yield, however, can be achieved when in vivo-matured and -fertilized oocytes are cultured in vitro [4]. Despite the efficiency of IVEP in yielding considerable numbers of embryos, more efforts are needed to improve the quality of these in vitro-produced embryos. Many features differ between in vitro and in vivo bovine embryos (for review see [5] and [6]). Darker cytoplasm, lower density [7], swollen blastomeres [8], slower growth rate, and higher thermal sensitivity [9] make in vitro-produced embryos weaker and more sensitive to cryotechnologies, and they yield a lower pregnancy rate [10] than in vivo embryos. Iwasaki et al. [11] found that in vitro-produced embryos had a lower ratio of inner cell mass (ICM) to trophectoderm (TE) cells, together with a less compact ICM in relation to both in vivo-produced embryos and embryos matured and fertilized in vitro but cultured in vivo. Moreover, in vitro-produced embryos showed a higher incidence of cytogenetical abnormalities [12] in relation to in vivo-produced embryos [13].

Gap junctions are channels in the plasma membrane of cells that allow the passage of ions and small molecules between neighboring cells [14, 15]. Since they transfer developmental information, they may play a role in the regulation of early embryonic development. Evidence to this effect is provided by findings that 1) gap junctional compartments are created in embryos at particular developmental stages [16, 17]; 2) junctional conductance changes at developmentally significant times [18, 19]; 3) defective embryos are generated after micro-injection of anti-gap antibodies or antisense RNA for gap junction molecules [19–21].

The time of appearance of gap junctions in the embryo varies between species. In the amphibian embryo, *Xenopus laevis*, functional gap junctions appear around the 4th cleavage [22]. In the sea urchin embryo, electron microscopy studies showed gap junction-like structures at the 16-cell stage [23]. In both cases it is not clear whether coupling is regionalized or is between all cells. Blastomeres in the ascidian embryo are electrically coupled at the 2-cell stage and dye coupled from the 32-cell stage [24, 25]. In mouse embryos, compaction at the 8-cell stage seems to be a key stage at which electrical and dye coupling are established via gap junctions [26], while in the human embryo, gap junctions appear later at the blastocyst stage between all cell types [27].

Cell polarity in early blastomeres seems to be a fundamental event for compaction and subsequent formation of the TE and ICM and blastocoel expansion [28], and these processes depend on the formation of other intercellular devices such as tight junctions [28, 29]. Prather and First [30], using dye-coupling technology, showed that blastomeres in bovine embryos were less intensely coupled than murine embryos and that blastomeres in in vitro bovine embryos were not coupled. Recently, Wrenzycki et al. [31, 32] examined the presence of mRNA encoding for connexin 43 in bovine embryos. Transcripts were found in in vitro-produced embryos up to the 8-cell stage in in vivo morulae and blastocysts, but not in in vitro morulae and blastocysts. These experiments suggest a defective transcriptional activity in in vitro-produced bovine embryos. The aim of the present study was to identify differences in cell-to-cell communication in in vitro and in vivo bovine embryos.

MATERIALS AND METHODS

Materials

If not otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

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[1]Correspondence: R. Boni, Dipartimento di Scienze delle Produzioni Animali, Via N. Sauro, 85, 85100 Potenza, Italy. FAX: 39 0971 470719; e-mail: boni@unibas.it

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In Vivo-Produced Embryos

Holstein Friesian and Brown Swiss cows were superovulated at Day 10 of the estrous cycle (estrus = Day 0) using 750 IU porcine FSH (Pluset, Serono, Italy) administered twice daily for 5 days using a decreasing schedule (250, 200, 150, 100, and 50 IU). On the third day of treatment, a single dose of prostaglandin $F_{2\alpha}$ was administered. At standing estrus and 12 h later the cows were artificially inseminated, and 5–8 days later, morula and blastocyst stage embryos were collected nonsurgically. Some of the recovered embryos were fixed immediately, and others were transferred to Tissue Culture Medium 199 (TCM199) + 10% FCS + 10 mM Hepes and transported to the lab within 4–5 h of collection, using a minincubator (K-Systems, Birkerod, Denmark) at 38.5°C.

In Vitro-Produced Embryos

Ovaries from slaughtered cows were collected from the abattoir and transported in a thermal bag at 30–35°C to the laboratory within 3–4 h of collection. The laboratory temperature was 30°C. Immature oocytes were collected from 2- to 8-mm follicles by an 18-gauge needle under controlled pressure [33]. Cumulus-oocyte complexes (COC) were isolated from the follicular fluid and washed 3 times with TCM199 + 5% FCS + 10 mM Hepes. The COC were then transferred into maturation medium (i.e., TCM199, 10% FCS, 10 IU/ml LH, 0.1 IU/ml FSH, 1 μg/ml estradiol-17β) (40 μl/COC) and left in an incubator at 39.0°C in 5% CO_2 humidified air. Twenty-four hours later, the COC were transferred in Fert-TALP medium (10 μl/COC) [34]. Frozen bovine sperm from an in vitro fertilization (IVF)-tested bull was thawed and then centrifuged at 1000 rpm for 30 min using a discontinuous Percoll gradient (90:40). After two washes in Hepes-TALP [34], the spermatozoa were incubated for 15 min with 100 IU/ml heparin and then added to the COC in the Fert-TALP at a final concentration of 2×10^6/ml (10^4 spermatozoa/COC). Two days later, the fertilized oocytes were transferred into 1 ml of hyaluronidase (Medi-Cult, Copenhagen, Denmark) and freed from the cumulus cells and the attached spermatozoa by vortexing for 2 min. Embryos at 2- to 8-cell stages were then cultured in SOFaaBSA [35] in a gas mixture of 5% CO_2, 7% O_2, and 88% N_2 [36] at 39°C. The media of in vitro-cultured embryos were covered with embryo-tested oil (Medi-Cult), and the culture dishes (Nunclon, Nunc, Roskilde, Denmark) were changed every 3 days. In vitro culture was carried out until Day 9 postinsemination (pi).

Physiological Techniques

Electrical and dye coupling between blastomeres were studied at several stages of embryo development in vitro, e.g., 2-cell (26–28 h pi), 4- to 8-cell (40–44 h pi), morula (5–6 days pi), blastocyst (7–8 days pi), and hatched blastocyst (8–9 days pi) stages and in vivo, e.g., 16-cell, morula, blastocyst, and hatched blastocyst. Only the most advanced embryos at each developmental stage of culture were used in our experiments. At each stage, experiments were repeated 5 times.

Before micromanipulation, the zona pellucida was removed by incubating the embryos in 0.5% Pronase for 1.5–2 min (in vitro embryos) or 5–7 min (in vivo embryos). After treatment, the zona-free embryos were incubated in IVF medium (Medi-Cult) for 30 min before use. Zona-free embryos with normal morphology were placed in a recording chamber containing 2 ml of Ham's F-10 (Mascia Brunelli, Milano, Italy) and kept at 38°C.

ICM from both in vivo and in vitro expanded and hatched blastocysts were isolated by the following microsurgical technique: zona-free embryos were placed in Ham's F-10 within a chamber under a traditional inverted microscope (Nikon, Garden City, NY) and, using a microblade (Sharpoint; Surgical Specialties Co., Reading, PA) mounted on a Narishige (Tokyo, Japan) manipulator, were cut to grossly separate the ICM from the TE.

Blastomeres were randomly selected for patch clamp and dye injection.

Electrical Coupling

Two standard patch micropipettes were used in the double whole-cell voltage clamp configuration to voltage clamp the two blastomeres. Electrodes of about 10 Mohm resistance and 1- to 2-μm-tip diameter were filled with an intracellular-like solution (ICS) medium composed of 70 mM KCl, 7 mM NaCl, 10 mM EGTA, 10 mM Hepes, pH 7.4, and 280 mOsm. By using standard techniques [24] we obtained gigaohm seals on two cells, set the pipette voltage at −20 mV, and ruptured the patches. Access to the cytosol was assessed by measuring a stable negative resting potential. Depolarizing and hyperpolarizing voltage steps in ramps of 10-mV steps and 500 msec duration were applied to one cell to generate junctional currents. Data were stored on VCR tape and subsequently analyzed by the General Linear Models procedure of SAS [37]. Junctional conductance (Gj) was calculated from junctional current/junctional voltage (Ij/Vj) measured during a series of voltage clamp pulses across the intercellular junctions.

Dye Coupling

Standard micropipettes were used to inject Lucifer Yellow (5% in ICS medium) (excitation = 428 nm; emission = 531 nm) into one blastomere [38], also in the whole-cell configuration, using an Eppendorf (Hamburg, Germany) microinjector. After injection, embryos were observed for 15 min under an epifluorescence Nikon inverted microscope and photographed.

Electron Microscopy

In vivo and in vitro embryos were prepared for transmission electron microscopy (TEM) analysis according to the methods of Dale et al. [27], at various stages of their development. In brief, the embryos were fixed in 3% glutaraldehyde, in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 4°C. Washed in the same buffer, the samples were postfixed in 1% osmium, 0.8% K3Fe(CN6) (Taab Laboratories Equipment, Reading Berks, UK) in buffer for 1 h at 4°C. After a quick wash in distilled water, they were stained en bloc in 0.5% uranyl acetate for 1 h at room temperature. After dehydration, embryos were embedded in Epon 812 (Electron Microscopy Sciences, Fort Washington, PA). Semithin sections were stained with 1% toluidine blue in 1% sodium borate. Serial thin sections were cut with diamond knives on an Ultracut (Leica Ultracut uct, Wetzlar Gmbh, Germany) microtome, collected on Formvar (Formvar Solution EM, Taab)-coated and uncoated grids, and stained with alcoholic uranyl acetate and Reynolds (Taab) lead citrate. Thin sections were observed with a Philips TEM 400 (Philips Eindhoven, The Netherlands).

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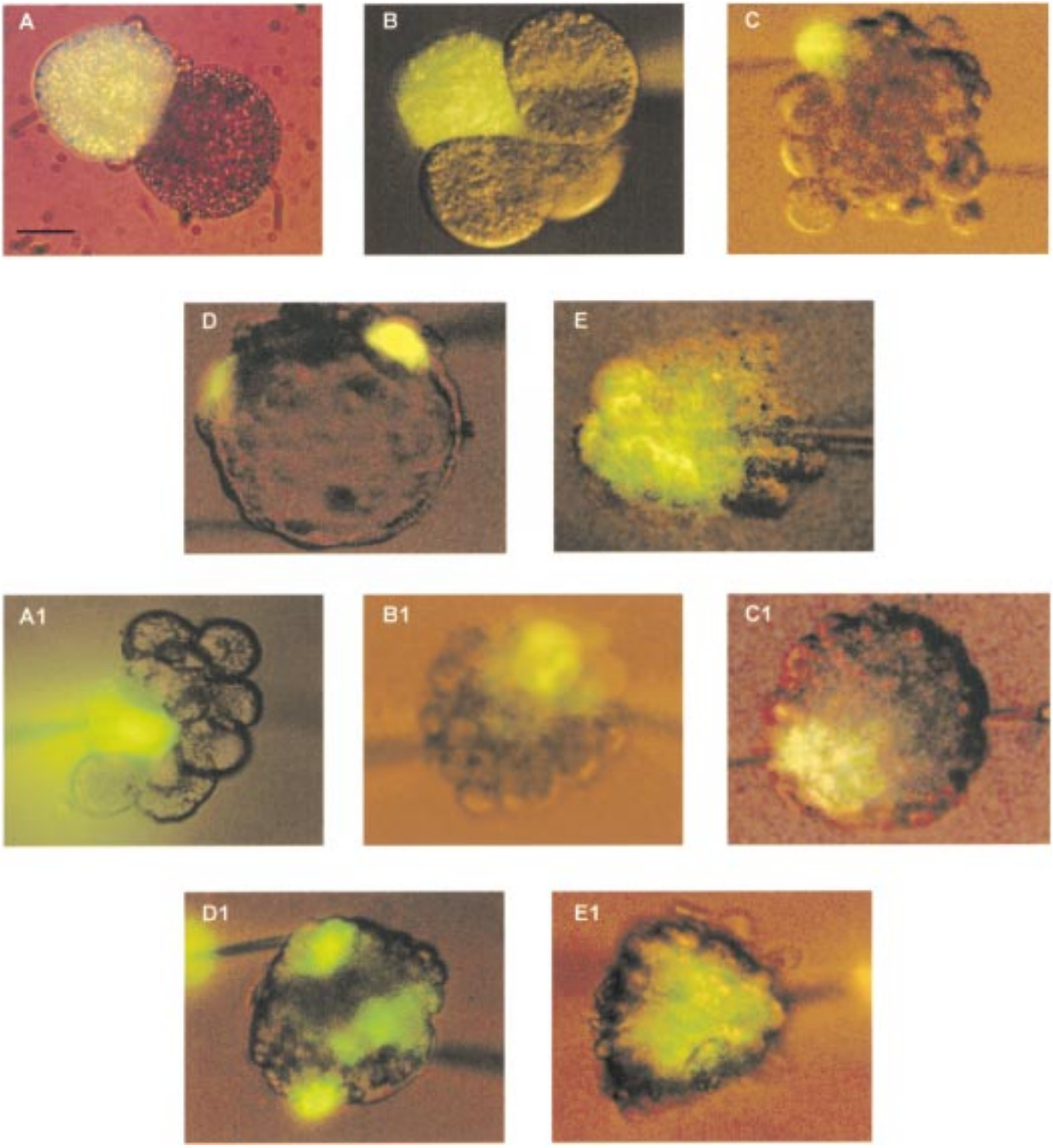

FIG. 1. Half-light and half-fluorescence photographs of bovine embryos at different stages of development after injection of Lucifer Yellow. Top panels (**A**-**E**) show the in vitro-produced embryos. No dye coupling was observed in the 2-cell (**A**), 4-cell (**B**), morula (**C**), and blastocyst (**D**) stages. A partial diffusion was found in the ICM (**E**). Bottom panels (**A1**-**E1**) show the in vivo-produced embryos. A lack of diffusion was found in the 16-cell stage (**A1**). Dye coupling started at morula stage (**B1**). In blastocysts there was a partial diffusion only when injection was performed in the ICM area (**C1** and **D1**). The isolated ICM showed a complete diffusion (**E1**). Note: The dark ring surrounding the ICM may be attributed to the cytolysis of the external blastomeres following microsurgery. Bar = 30 μm.

RESULTS

The IVEP efficiency in our laboratory was 78% cleavage rate and 28% expanded blastocyst yield at Day 8 pi. The mean (± SD) number of nuclei was 99 ± 10 in Day 7 expanded blastocysts (n = 10) and 151 ± 12 in Day 9 hatched blastocysts (n = 10).

Lucifer Yellow injected into a blastomere of in vitro embryos filled the blastomere within 10 sec but never spread

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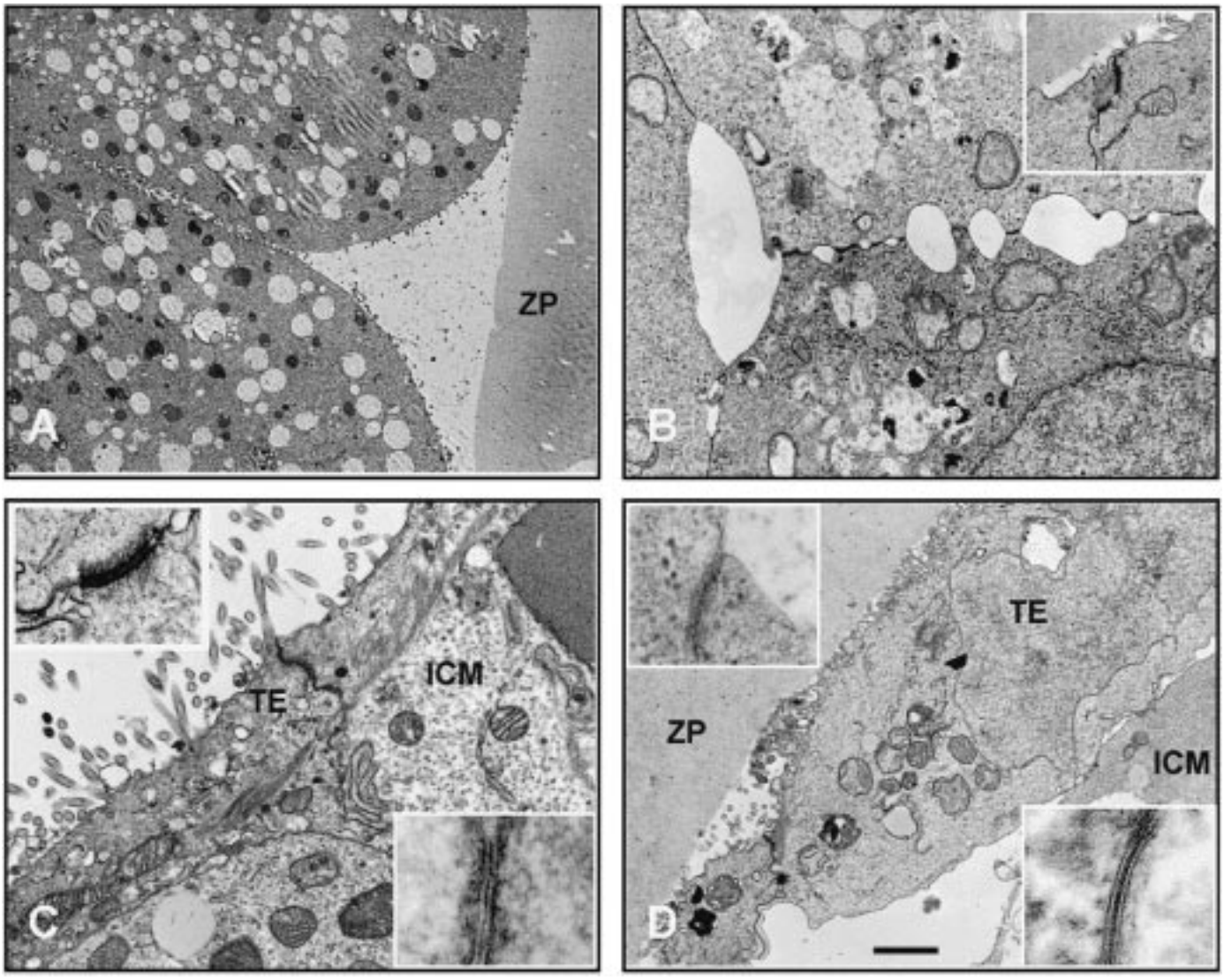

FIG. 2. Transmission electron micrographs (published at 96%) of adjacent blastomeres in bovine embryos at different stages of development. **A**) In vitro-produced 2-cell embryo (×4300). The apposed cell surface shows microvilli and not communicative or adhesive structures. **B**) Three adjacent cells in an in vivo morula (×8800). Tight junctions and desmosome-like structures (inset, ×22 400) are present both in vivo and in vitro. **C**) In vitro-hatched blastocyst (×11 000). Tight junctions and desmosome-like structures (upper inset, ×17 000) were found in the TE, whereas gap junctions (lower inset, ×134 000) appeared in ICM. The microvilli were on the apical surface of the TE cells. **D**) In vivo blastocyst (×5700). Tightly opposed contact areas (upper inset, ×29 000) were present in both the ICM and the TE. Higher-density intercellular devices such as desmosome-like structures and gap junctions (lower inset, ×173 000) were found in vivo with respect to the in vitro stage. Note: Insets show structures that were not always from the main photograph but from surrounding areas.

to neighboring blastomeres, at all stages studied from the 2-cell stage to the blastocyst stage (Fig. 1, A–C). In blastocysts, the dye remained limited to the blastomere injected (Fig. 1D), except for one experiment in which it diffused to a few neighboring cells. In contrast, in isolated ICM, Lucifer Yellow spread from the injected cell to the other cells except for a few remaining attached cells, maybe of TE origin (Fig. 1E). In the case of in vivo embryos there was no diffusion at the 16-cell stage (Fig. 1, A1), and a partial diffusion to a few cells occurred in morulae and blastocysts (Fig. 1, B1, C1, and D1). Isolated in vivo ICM from both expanded and hatched blastocysts showed complete passage of the dye (Fig. 1, E1).

Gap junctions were not observed by TEM in in vitro embryos until the blastocyst stage. From the 2- to the 8-cell stages, apposed plasma membranes were organized into microvilli (Fig. 2A). Tight junctions were first observed at the morula stage. In in vitro-produced blastocysts, gap junctions were found in the ICM as well as between ICM and TE, but not between TE cells. Zonula adherens and tight junctions were abundant in the blastocyst. The density of gap junctions between ICM increased in the hatched blastocysts (Fig. 2C).

In the in vivo embryos, rare gap junctions appeared at the morula stage. Tight junctions at this stage were more evident (Fig. 2B); a high density of gap junctions was found at the blastocyst stage (Fig. 2D), with a higher density in the ICM compared to the TE cells.

The resting potential of bovine blastomeres ranged from −1 to −54 mV (14.6 ± 11.5 mV) with no appreciable differences between the stages or with respect to their in vivo or in vitro origin (14.7 ± 11.4 vs. 14.4 ± 11.8 mV). Using the dual voltage clamp technique and applying rectangular voltage pulses across the junction from a potential of −20 mV, we found in the case of in vitro embryos a Gj decreasing from 4.18 ± 1.70 nS at the 2-cell stage to 0.37

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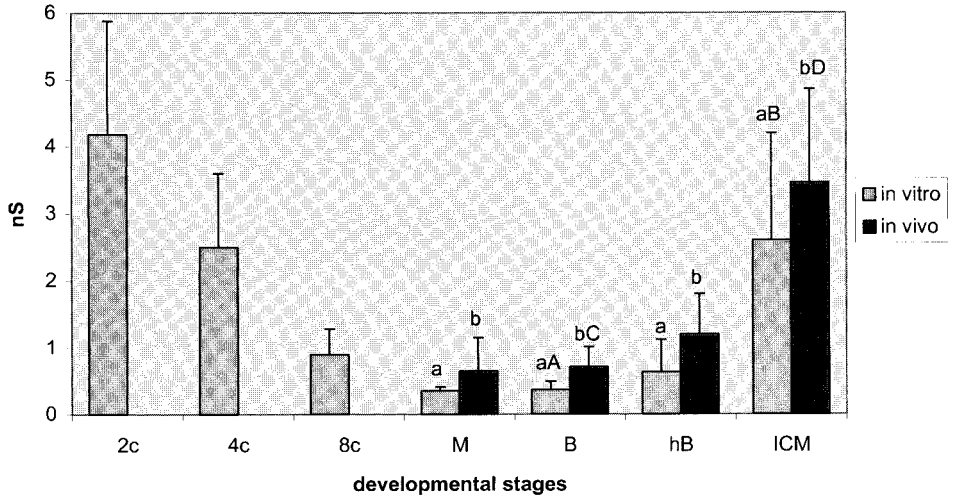

FIG. 3. Cell-to-cell conductance in in vitro- and in vivo-produced embryos at different stages of development, e.g., 2-cell (2c), 4-cell (4c), 8-cell (8c), morula (M), blastocyst (B), hatched blastocyst (hB) stages, and in ICM microsurgically obtained from expanded blastocysts. a,b ($P < 0.05$); A,B and C,D ($P < 0.01$).

$\pm$ 0.12 nS at the blastocyst stage (Fig. 3). We have no data for the early in vivo stage. At each examined developmental stage, in vivo embryos showed a significantly ($P < 0.05$) higher Gj than in vitro embryos. In both in vivo and in vitro embryos a significant increase was, however, found at the hatched blastocyst stage, particularly in the ICM. Isolated ICM from both in vitro and in vivo blastocysts had a transjunctional inter-blastomere conductance significantly ($P < 0.01$) higher than intact blastocysts (2.6 $\pm$ 1.6 vs. 0.37 $\pm$ 0.12 nS for the in vitro and 3.5 $\pm$ 1.4 vs. 0.71 $\pm$ 0.3 nS for the in vivo; $P < 0.01$). Electrical communication was inhibited with the addition of 2.5 mM 1-octanol to the bath.

DISCUSSION

Using a low molecular weight fluorescent dye, Lucifer Yellow, we have shown that blastomeres in in vivo- and in vitro-produced early embryos are coupled by functional gap junctions. Dye coupling was first observed at the morula stage in the in vivo embryos. At the blastocyst stage, differences were found in relation to the origin of the embryos; in fact, in vivo embryos showed a partial intercellular communication whereas in the case of in vitro embryos the dye never spread throughout the embryo (Fig. 1, D vs. D1). This partially agrees with findings of Prather and First [30], who in a preliminary study on dye coupling in cattle embryos observed a partial diffusion of the dye in only 50% of in vivo blastocysts and 0% in the case of in vitro-matured and -fertilized and in vivo-cultured embryos. The partial diffusion of dye in embryos prompted us to isolate the 2-cell lines of the blastocyst, i.e., the ICM and the TE, and to inject dye into them separately. ICM showed good communication in both in vitro and in vivo embryos, whereas TE showed only partial communication in in vivo embryos and an absence of communication in the case of in vitro embryos. This finding may explain the results of Prather and First [30] in in vitro blastocysts, since they injected dye only into the TE cells. Although Wrenzycki et al. [31, 32] suggested a defective transcriptional activity for connexin 43 in the in vitro-produced morula and blastocysts, very recent studies from the same authors [39] supported our results, demonstrating a developed connexin 43 transcription also in the in vitro morulae and blastocysts under different culture conditions.

Ultrastructural analyses supported the dye-coupling observations. The absence of cell communication devices from the 2- to 16-cell stage in in vivo embryos was previously described by Brackett et al. [40]. The low density of tight junctions in in vitro morulae could be responsible for the lower degree of compaction found in these embryos [41]. In fact, tight junction development plays a crucial role in compaction [28], as well as in further embryo differentiation, i.e., ICM and TE. Also, the flattening of the outer cells observed in mouse [42] and human [28] embryos during compaction representing the beginning of TE differentiation is less evident in in vitro than in vivo embryos (data not shown). In vivo-produced bovine blastocysts had a low density of gap junctions [41], while a higher density was found by Mohr and Trounson [42] in hatched blastocysts. Shamsuddin et al. [43] showed a lower number of junctional structures in in vitro than in in vivo embryos, and this is supported by our study.

In in vitro-produced bovine embryos, electrical coupling between blastomeres is established at early stages and is decreased to the blastocyst stage. The same pattern has been observed in ascidian [24] and sea urchin [44] embryos. This progressive decrease of the Gj from the 2-cell stage to the advanced stages may be attributed to the architecture of the embryo. We attempted to quantify the difference in junctional communication in in vitro and in vivo embryos by measuring the Gj using the whole-cell voltage clamp technique. The higher Gj values found in the in vivo embryos and in the ICM from both in vivo and in vitro embryos demonstrated that this parameter is correlated to gap junction development, and supported our findings from dye test and electron microscopy. At early stages of bovine embryo development, however, Gj values were not correlated to dye test and electron microscopy. This finding in the bovine embryos reflects the situation known in the ascidian and sea urchin embryos. Since the electrical coupling is not due to cytoplasmic bridges [25, 27, 44], it is possible that early embryos express a ''maternal''-type gap junction that is replaced in bovine embryos at the blastocyst stage, similar to the situation in ascidian embryos [24]. In contrast, in the mouse embryo Lo and Gilula [45] found a good correlation between electrical and dye coupling at early developmental stages. Finally, the difference in cell-to-cell communication between TE and ICM may reflect the different roles of these two cell lines in that the TE cells are in effect an epithelial-like barrier, while the ICM needs intercellular communication devices in order to coordinate the ensuing processes leading to early embryogenesis.

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